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Gastric Digestion of Bovine Lactoferrin In Vivo in Adults¹

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Freddy J. Troost,^{*2} Jan Steijns,[†] Wim H. M. Saris^{*} and Robert-Jan M. Brummer^{**}

^{*}Department of Human Biology, Nutrition and Toxicology Institute Maastricht, Universiteitssingel, Maastricht, The Netherlands; [†]DMV International, Center of Expertise Nutrition, Bornsesteeg, Wageningen, The Netherlands; and ^{**}Department of Gastroenterology, Nutrition and Toxicology Institute Maastricht, University Hospital Maastricht, AZ Maastricht, The Netherlands

ABSTRACT Lactoferrin (LF), an iron-binding glycoprotein present in milk and other endocrine and exocrine secretions, may exert a number of physiologic effects in the intestines. To study the effects of oral LF supplementation in vivo in the gastrointestinal tract, information about the gastric survival of LF in vivo is important. We tested 12 healthy volunteers (age 21 ± 0.3 y) on 3 separate d according to a randomized, cross-over design. A test drink containing 4.5 g of bovine LF (20% iron-saturated LF; apoLF) in the presence of a gastric pH buffer (0.1 mol/L sodium citrate/citric acid; apoLFbuf), apoLF without the buffer (apoLF) or iron-saturated LF (holoLF) was administered into the stomach using nasogastric intubation. Gastric emptying rate, determined by a marker dilution technique, did not differ among any of these drinks. Gastric survival of LF, analyzed by gel permeation chromatography under denaturing conditions, was 64%, 62% and 79% after consumption of the apoLFbuf, apoLF and holoLF test drinks, respectively. Addition of the gastric pH buffer initially lowered intragastric pH because of its hydroxide buffering effect. However, it did not elevate intragastric pH over a prolonged period and thereby inhibit intragastric LF breakdown. We conclude that after oral administration, substantial amounts of apoLF and holoLF survive gastric transit. *J. Nutr.* 131: 2101–2104, 2001.

KEY WORDS: • lactoferrin • breakdown • gastric emptying • biological activity • stomach

Lactoferrin (LF)³ is a protein of the transferrin family. It is present in milk, saliva, tears, bile, blood plasma and mucosal and genital secretions (1,2). LF binds ferric iron (Fe^{3+}) with a high affinity, even in an acidic environment (3,4). Because of

this strong iron-binding capacity, a number of physiologic effects have been proposed, as reviewed extensively by several investigators (2,4–6).

LF has been proposed to act as an antioxidant (7–9). Iron bound to LF may not be able to catalyze Fenton chemistry. This, however, has not yet been investigated in vivo. LF is also involved in the immune system of the body. It is released by neutrophil-specific granules (7). Other cells of the immune system, macrophages and monocytes, can bind LF by specific LF receptors on their cell surfaces, which enable the presence of LF at sites where the immune system is active. Several studies have shown a regulatory function of LF on interleukin-6 and tumor necrosis factor- α in vivo in mice (10,11). In addition, LF was shown to exert antibacterial activity in vivo (12,13) in mice and recently, LF ingestion with food was shown to facilitate a recovery from dermatophytosis in guinea pigs (14). LF has been proposed to serve a role in facilitating iron absorption from the gut by a mechanism that is still poorly understood. Previously, both a facilitating effect (15) and an inhibitory effect (16) of LF on iron absorption were described. These findings indicate that LF supplementation may be of value in patients prone to intestinal damage, such as people undergoing oral iron therapy, patients with inflammatory bowel disease or patients suffering from intestinal stress in general.

The effects of oral LF supplementation could be potentially diminished to a certain extent by gastrointestinal breakdown of the LF molecule. It is not known to what extent LF is digested in the stomach or in the intestines in vivo. In vitro incubation of LF with proteolytic enzymes results in the formation of several LF fragments. It was shown that LF is less rapidly digested in gastric juice than casein and transferrin in an acidic environment (17). An important factor interfering with this degradation process is the degree of iron saturation of the LF. Twenty percent iron-saturated LF (apoLF) is more easily digested than 100% iron-saturated LF (holoLF) (18,19). Because the biological activity of LF is located in the intestine, it is necessary to study the survival of the molecule during passage through the stomach.

The present study was designed to determine the extent of gastric degradation of LF in vivo. In addition, the effect of buffering gastric pH on the breakdown of LF was studied.

MATERIALS AND METHODS

Subjects. Twelve healthy volunteers (five men, seven women, age 21.3 ± 0.3 y) without a history of gastrointestinal complaints were recruited for this study. All subjects received full information about the protocol and gave their written informed consent before participation. This study was approved by the Ethics Committee of Maastricht University (Maastricht, The Netherlands).

Protocol. Subjects were tested according to a randomized, cross-over design with a washout period of at least 7 d. On the evening before testing, all subjects were instructed to consume a standardized meal ad libitum (rice and sweet/sour sauce; Uncle Ben, Veghel, The Netherlands). After an overnight 12-h fast, subjects arrived at the laboratory and remained seated until the end of the experiment.

After arrival, a nasogastric tube was inserted. The stomach was

¹ Supported by DMV International, Veghel, The Netherlands.

² To whom correspondence should be addressed. E-mail: f.troost@hb.unimaas.nl

³ Abbreviations used: apoLF, 20% iron-saturated LF; apoLFbuf, 20% iron-saturated LF in the presence of a gastric pH buffer; AUC, area under the curve; holoLF, 100% iron-saturated LF; LF, lactoferrin; PEG, polyethylene glycol.

emptied via the nasogastric tubing and rinsed with water until no additional residue was obtained. A recovery test was performed for control of the tube tip position (20). Forty-five minutes after gastric aspiration, a liquid test drink (composition described below) was administered into the stomach through the tube. Subsequently, the double sampling technique to determine gastric emptying rate of a test drink was applied. This method was described in detail previously (21–23). According to this technique, 188 mg of the inert marker polyethylene glycol (PEG; PEG 4000; Sigma, Deisenhofen, Germany) was added to each test meal. After ingestion of the test drink, gastric contents were mixed thoroughly by aspiration and reinjection of gastric contents for 1 min and 5 mL of gastric contents were sampled for measurement of total gastric content volume. At each 10-min interval, 2.5 mL of the gastric contents were aspirated and 5 mL PEG solution (60 g/mL water) were added to the gastric contents via the nasogastric tube. Subsequently, gastric contents were mixed thoroughly, and 2.5 mL were aspirated. This protocol was followed at 10, 20 and 30 min after administration of the test drink. The PEG concentration before and after addition of the PEG solution was measured. From these data, the total volume of gastric contents at each time point was calculated (23). Also, the amount of test meal still present in the stomach and the amount of gastric secretion per 10-min intervals was calculated using calculations as described by Beckers et al. (21). In the samples obtained at 10-min intervals before adding the PEG solution, the amount of intact LF was determined as described below. After aspiration, each sample was put directly into liquid nitrogen and stored at -80°C until analysis to stop degradation processes after sampling.

Test drinks. Three test drinks were investigated in a randomized, cross-over order. Each beverage consisted of 300 mL of 80 g/L maltodextrin (MD20; AVEBE, Veendam, The Netherlands) solution in water with 4.5 g bovine LF (DMV International, Veghel, The Netherlands) dissolved. The LF had an iron saturation of $\sim 20\%$ (apoLF) and 100% (holoLF). ApoLF was ingested on two occasions; once without a buffer (apoLF) and once in the presence of citrate to buffer gastric pH (apoLFbuf). The citrate buffer was prepared by mixing 0.1 mol/L citric acid monohydrate with 0.1 mol/L trisodium-citrate dihydrate in a volume ratio of 56:44 mL/L test drink, respectively. Citric acid was purchased from Merck (Darmstadt, Germany) and trisodiumcitrate was purchased from Ferak (Berlin, Germany).

Analytical methods. PEG concentration in the gastric juice samples was determined spectrophotometrically using a spectronic 1001 device (Bausch en Lomb, Bergen op Zoom, The Netherlands). Carbohydrates and proteins were removed by precipitation. In the test drinks and in each sample, pH was measured using a standard pH analyzer (Radiometer, Copenhagen, Denmark).

A new method was developed and validated to measure LF and LF digestion fragments in gastric samples. This method uses gel permeation chromatography under denaturing conditions. We found that LF degradation may proceed on the column in samples containing a relatively large proportion of gastric juice compared with residual test drink. When the sample contained $> 50\%$ gastric juice, the elution from the column had to occur within 5 h to prevent LF degradation by gastric pepsin at the low pH of the elution buffer.

Sample pretreatment: 100 μL gastric juice or LF standard were mixed with 450 μL 8 mol/L urea (Merck), pH 8.5. After solubilization, 450 μL of a mixture of H_2O /acetonitrile/trifluoroacetic acid (600/400/1 v/v) was added. The resulting solution was then filtered over 0.2 μm PVDF filter (Gelman Sciences, Pall Gelman, Portsmouth, ME). Twenty microliters was subsequently applied to the column (Shodex KW-803; Showa Denko, Tokyo, Japan) and eluted with the mixture of water/acetonitrile/TFA in 25 min at a flow rate of 0.6 mL/min (high performance liquid chromatography; Pharmacia LKB, Piscataway, NJ). Proteins and peptides were detected at 220 nm. Intact LF eluted after ~ 11 min, whereas LF fragments eluted later. Calibration of counts for intact LF was performed with concentrations between 1.3 and 13 g/L and a straight line was obtained. LF fragments between 9 and 80 kDa fitted a straight line obtained with protein molecular mass markers (LF, bovine serum albumin, β -lactoglobulin, α -lactalbumin and glycomacropeptide).

Calculations. Calculations of gastric emptying and secretion rates were based on the formulas proposed by Beckers et al. (21). For

these calculations, PEG concentrations in the gastric juice samples were used to determine gastric content volume at each time point and gastric secretory rate at 10-min intervals. A gastric emptying curve was constructed according to a nonlinear regression formula using specialized software (GraphPad Prism, San Diego, CA). From this curve, gastric emptying half time of the test drink was determined.

The amount of intact LF entering the intestine was determined by calculating the area under the curve (AUC) of the gastric emptying curve and gastric LF concentration. The AUC of the gastric emptying curve was considered to represent emptying of the ingested amount of 4.5 g LF into the small intestine. Hence, gastric LF breakdown was calculated by subtracting the AUC of LF breakdown from the AUC of the gastric emptying curve. LF delivery to the intestines was calculated by subtracting the gastric LF breakdown from the ingested amount of 4.5 g LF.

Statistics. Differences in gastric emptying and in gastric pH were analyzed using one-way ANOVA for repeated measures with Scheffé posthoc testing. Differences in LF appearance in the small intestine among the three drinks were analyzed using ANOVA with the amount of LF as the dependent variable, test drink as factor and volunteer as covariate. Differences were considered significant at the probability level of $P < 0.05$. All statistical analyses were carried out using Statview software (SAS Institute, Cary, NC). Values are means \pm SD.

RESULTS

Gastric emptying rate is expressed as gastric emptying half time ($t_{1/2}$) of the test drink. Gastric emptying half times were 6.79 ± 2.36 , 8.46 ± 4.07 and 7.03 ± 2.14 min for apoLFbuf, apoLF and holoLF, respectively. Gastric emptying rates did not differ among any of the test drinks.

The initial pH of the apoLF test drink in the presence of the pH buffer was 5.95, of the apoLF solution without buffer, 7.12, and of the holoLF solution without buffer, 7.10. Intra-gastric pH of the apoLF solution with the citrate buffer during the period of testing was significantly lower directly after ingestion of the test drink compared with after both the apo- and the holoLF solutions without buffer (Fig. 1). Ten minutes after ingestion, intragastric pH was still lower after ingestion of the apoLF solution with buffer compared with the holoLF, but not compared with the apoLF drink. Twenty and thirty minutes after administration, no differences were observed in intragastric pH after consumption of any of the test drinks. In

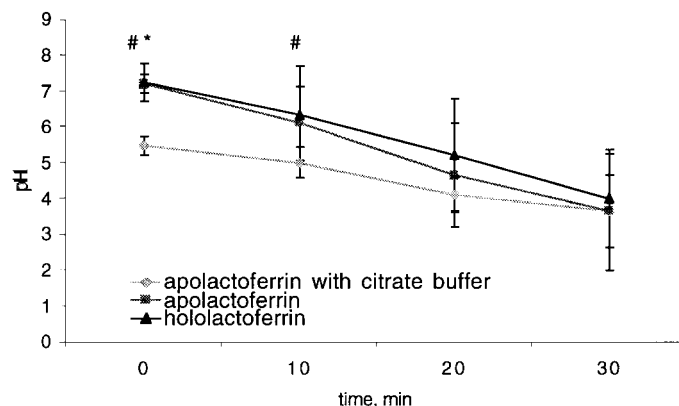


FIGURE 1 Intra-gastric pH in humans after ingestion of 4.5 g bovine apoLF with citrate buffer (drink 1), 4.5 g bovine apoLF without buffer (drink 2) and 4.5 g bovine holoLF without buffer (drink 3). All drinks contain 80 g/L maltodextrin in 300 mL water. Values are means \pm SD, $n = 12$. * $P < 0.05$, drink 1 vs. drink 2; # $P < 0.05$, drink 1 vs. drink 3.

addition, no differences in pH were observed at any time among the nonbuffered test drinks.

After 30 min, all test drinks were nearly completely emptied into the intestine. During this period, 64, 62 and 79%, respectively, of the LF present in the apoLFbuf, apoLF and holoLF test drinks entered the intestine in the intact form (Fig. 2). No differences in LF survival were observed among any of the test drinks, although the holoLF solution tended to be more resistant to degradation than the apoLF solution ($P = 0.09$).

DISCUSSION

All test drinks were emptied from the stomach at the same rate. Within ~10 min, half was already emptied. Because of this rapid gastric emptying of the LF during which only a small portion of the LF was digested, a large amount of LF entered the small intestine in the intact form.

Iron binding was shown previously to stabilize the bovine and human LF molecule (18,19), causing holoLF to be less susceptible to degradation than apoLF. This was shown after incubation with trypsin and chymotrypsin. We did not observe a significant difference in degradation rate among any of the test drinks, although holoLF tended to be more resistant to degradation than apoLF ($P = 0.09$). This indicates that the observed difference in susceptibility to degradation between apo- and holoLF from these *in vitro* studies is not reflected in the same manner in our *in vivo* study of intragastric degradation of LF, in which pepsin probably is the major cause of LF degradation. The apoLF used in the present study was 20% iron-saturated. Considering that iron binding to LF diminishes the susceptibility of LF to digestion, apoLF containing no iron may be digested in the stomach more rapidly than the apoLF used in the present study.

Other investigations showed a minor degradation of LF both *in vivo* in the entire digestive tract in newborns (24) as well as *ex vivo* in gastric juice of preterm infants (17), although the latter study did show some degradation. In the present study, we showed partial LF degradation in the adult gastrointestinal tract *in vivo*.

Intragastric pH was higher during the first 10 min after ingestion of both the apo- and the holoLF drink without citrate buffer compared with the apoLF solution with a 0.1 mol/L sodium citrate buffer. The buffer kept the intragastric pH relatively constant throughout the test. After 20 min, most of the citrate buffer was emptied from the stomach with the rest of the beverage, causing a decrease in buffering capacity and consequently, intragastric pH was determined mainly by gastric acid secretion. From *in vitro* work performed at our laboratory, we know that LF is resistant to degradation at a pH

above 4. We observed this in an experiment in which we incubated LF in gastric juice *ex vivo*. This can partly be explained by the previous finding that iron is released from LF at pH lower than 4.0 (25). Iron stabilizes the LF molecule. Thus, iron release causes the LF molecule to be more prone to digestion (18,19). Addition of the citrate buffer did not prevent gastric breakdown of LF in the present study because the buffer was emptied before it could effectively prevent additional acidification of gastric contents. Although gastric pH did not fall below 4.0 until the last 10 min of the experiment, when most of the test drink was already emptied from the stomach, we observed a considerable LF breakdown in the stomach. Hence, intragastric LF digestion *in vivo* occurs also at a pH higher than 4.0. This indicates a difference between the results of *in vitro* and *in vivo* experiments.

Intact LF, which has a size of ~79 kDa, consists of two lobes: the C- and the N-lobe. Incubation of LF with pepsin leads to formation of a 39.5-kDa fragment, most likely the C-terminal half of LF (26). After incubation of LF *ex vivo* in gastric juice of preterm infants, LF fragments of 33, 34, 41 and 42 kDa were formed, probably also representing half-molecules of the LF molecule (17). The N-terminal portion of LF was more prone to digestion by pepsin than the C-terminal half (27). After incubation with pepsin, the remaining C-terminal portion of the LF molecule still has iron-binding capacity. However, after incubation with trypsin and chymotrypsin, two fragments were obtained; the C-terminal and the N-terminal portions of LF (26). Both of these LF fragments are still able to bind iron ions. In other studies, *in vitro* incubation of LF with trypsin causes the LF molecule to break down to fragments with molecular sizes of 20, 30, 40 and 50 kDa (28). In addition, after LF ingestion in adult mice, LF fragments containing a peptide called lactoferricin were detected in the feces of these mice. Lactoferricin, also formed after hydrolysis with pepsin, is a biologically active component of the LF molecule of ~3.2 kDa. It was shown to have bactericidal activity against Gram-positive and, especially, against Gram-negative bacteria (29,30). The lactoferricin shows a stronger antibacterial activity than the intact LF molecule, indicating a functional role of gastric hydrolysis for optimal biological activity (29).

In our study we found LF fragments of several different sizes at 10 min after ingestion of the test drinks. Most abundant were fragments with a molecular mass of 76 and 41 kDa, although we did not quantify these fragments. The presence of the 76-kDa fragments indicated a rapid hydrolysis of small parts of the LF molecule. In the present study, we did not regard LF molecules of 76 kDa or smaller as being intact LF because any biological activity of intact LF may be different from the 76-kDa fragments. The 41-kDa fragment probably is the C-terminal portion of the LF molecule. We did not examine the precise nature of the fragments. With radial immunodiffusion, we established that immediately after drinking the test drinks, the recovery of intact LF was 100% (data not shown). When digestion of LF occurred, as evidenced by the high performance liquid chromatography pattern, we could demonstrate that LF fragments larger than 35 kDa still were able to bind to the antibodies directed against the intact LF (data not shown).

In this study, LF was intragastrically administered and 45 min before administration, the stomach was rinsed. This was necessary for the protocol used to measure gastric emptying and tube tip control. During the 45 min before test drink ingestion, subjects remained seated. Considering a normal average gastric juice production of 1500 mL/d, 45 min is sufficient to restore normal resting gastric juice volume of 50 mL. Theoretically, it is possible that orally administered LF is

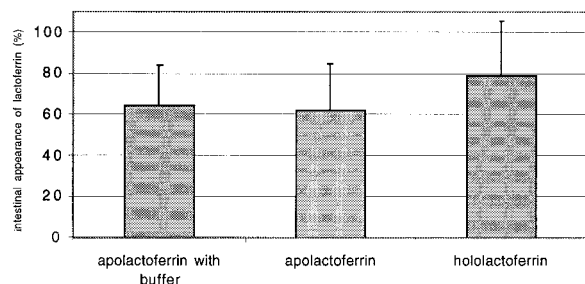


FIGURE 2 Total amount of intact bovine LF entering the small intestine of humans expressed as a percentage of the amount of bovine LF intragastrically administered. Values are means + SD, $n = 12$.

degraded differently from what we found in the present study, but we believe that the protocol reflects the normal physiological digestive environment.

A number of biological effects are attributed to LF, such as an antioxidative (7–9), an antiinflammatory, a bactericidal effect (29–33) and a facilitating role in iron absorption (15). To exert these effects, LF or biologically active LF fragments must survive passage through the stomach. To our knowledge, this is the first study to show that a major proportion of orally administered bovine LF survives passage through the stomach in adults. Intragastric degradation is not significantly affected by the iron saturation of the LF molecule and addition of a citrate buffer also did not influence breakdown. These findings are essential in interpreting results of studies concerning biological effects of LF in the gastrointestinal tract.

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